(REV 10/01) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 36 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
MBP-010XX	
U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	
10/070302	

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/08711	06 September 2000 (06.09.00)	06 September 1999 (06.09.99)

TITLE OF INVENTION

CONGENER INDEPENDENT DETECTION OF MICROCYSTIN AND NODULARIN CONGENERS

APPLICANT(S) FOR DO/EO/US

Daniel R. Dietrich, Werner Fischer, A. Richard Chamberlin, James B. Aggen, Ian Garthwaite, Christopher O. Miles, Kathryn M. Ross, and Neale R. Towers

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2)) **in ENGLISH**
   a. ☐ is attached hereto (required only if not transmitted by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(3)).
   a. ☐ is attached hereto.
   b. ☐ had been previously submitted under 35 U S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U S.C. 371(c)(4)).
10. ☒ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).
    Consisting of 4 sheets of Claims 1-18.

Items 11. to 20. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98. (+ copy of International Search Report)
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825
18. ☐ A second copy of the published international application under 35 U S C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☐ Other items or information:

Express Mail Number
EV 009952176 US

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/070302	PCT/EP00/08711	MBP-010XX

21. ☒ The following fees are submitted:

		CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):			
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1,040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	**$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$740.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$710.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)	**$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =		$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		$ 0	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	28 - 20 =	8	X **$18.00**	$ 144.00	
Independent claims	1 - 3 =	0	X **$84.00**	$ 0	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+**$280.00**	$ 0	
TOTAL OF ABOVE CALCULATIONS =				$1,034.00	
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$ 517.00	
SUBTOTAL =				$ 517.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 Months from the earliest claimed priority date (37 CFR 1.492(f)).				$ 0	
TOTAL NATIONAL FEE =				**$ 517.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property				$	
TOTAL FEES ENCLOSED =				$ 517.00	
				Amount to be Refunded:	$
				Charged:	$

a. ☒ A check in the amount of $ 517.00 to cover the above fees is enclosed. A check in the amount of $_____ is enclosed for the assignment recordation fee.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-0804 . A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

Customer Number 207
SEND ALL CORRESPONDENCE TO:

Weingarten, Schurgin, Gagnebin & Lebovici LLP
Ten Post Office Square
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SIGNATURE

NAME: Charles L. Gagnebin III
REGISTRATION NUMBER: 25,467

Date: 3-5-2

CLG/mc/268242-1

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application	:	DANIEL R. DIETRICH, ET AL.
Application No.	:	
Filed	:	Herewith
For	:	CONGENER INDEPENDENT DETECTION OF MICROCYSTIN AND NODULARIN CONGENERS
Examiner	:	
Attorney's Docket	:	MBP-010XX

Group Art Unit:

* *

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231 on _____.

By:_____
Charles L. Gagnebin III
Registration No. 25,467
Attorney for Applicant(s)

* *

PRELIMINARY AMENDMENT

BOX PCT
Commissioner for Patents
Washington, D.C. 20231

Sir:

Submitted for examination is the attached English translation of the claims that were amended by the International Preliminary Examination Report dated July 19, 2001.

Kindly enter the following Preliminary Amendment in the above-identified application:

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Express Mail Number
EV 009952176 US

Attorney Docket No. MBP-010XX
Filed: Herewith
Group Art Unit:

In the Claims:

In the Amended Claims of the International Preliminary Examination Report (attached) dated July 19, 2001, please amend the Claims to read as follows (a copy of the amended claims showing the additions and deletions appears at the end for the Examiner's convenience):

3. The compound according to claim 1, wherein the groups R^3 each represent methyl and group R^4 represents methoxy.

4. The compound according to claim 1, wherein group R^1 represents aminoacyl and group R^2 represents (C_1-C_4)acyl.

6. The compound according to claim 1, wherein group R^1 represents $-NH_2$ and group R^2 represents glutamidyl or 2-aminopropionamidyl.

7. The compound according to claim 1, wherein the toxin is selected from the group consisting of mycrocystin and nodularin congeners.

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8. The compound according to claim 1 which is a polyclonal, monoclonal or recombinant antibody or a functionally active derivative or fragment thereof.

9. A method for the preparation of the compound according to claim 1 comprising the steps of

(a) preparing a compound containing a group represented by formula (I) as defined in claim 1,

(b) coupling the compound of step (a) to a carrier.

13. The method according to claim 9 which further comprises the steps of

(c) immunizing an animal with the conjugate obtained in step (b), and

(d) isolating the animal's blood, blood serum and/or spleenocytes.

14. A diagnostic kit containing the compound according to claim 1.

15. An affinity matrix containing the compound according to claim 1 coupled to a polymeric resin.

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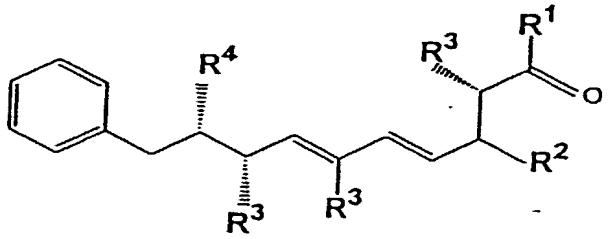
AMENDED CLAIMS PER INTERNATIONAL PRELIMINARY EXAMINATION REPORT DATED 19 JULY 2001

Claims

1. A compound comprising one or more polypeptides providing a binding site of a monoclonal, polyclonal or recombinant antibody or a functionally active derivative or part thereof for a group represented by the following formula (I)



(I)

which is part of a toxin derived from a cyanobacterium, wherein

group R^1 represents a halogen atom, $-OSO_3$, $-OR'$ or $-NR'_2$ and

group R^2 represents hydrogen, (C_1-C_4)alkyl, (C_1-C_4)alkoxy, (C_1-C_4)acyl, (C_1-C_4)aminoacyl or (C_1-C_4)carboxyaminoacyl, or the groups R^1 and R^2 are connected to each other to form a cyclic moiety,

the groups R^3 which may be the same or different are each independently selected from the group consisting of hydrogen and (C_1-C_4)alkyl,

group R^4 represents (C_1-C_4)alkoxy,

and wherein the phenyl group may be substituted or unsubstituted.

2. The compound according to claim 1, wherein the groups R' represent independently from each other hydrogen, substituted or unsubstituted (C_1-C_4)alkyl or (C_1-C_4)acyl, when bound to nitrogen.

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AMENDED CLAIMS PER INTERNATIONAL PRELIMINARY EXAMINATION REPORT DATED 19 JULY 2001

3. The compound according to claim 1 or 2, wherein the groups R^3 each represent methyl and group R^4 represents methoxy.

4. The compound according to any one of claims 1 to 3, wherein group R^1 represents aminoacyl and group R^2 represents (C_1-C_4)acyl.

5. The compound according to claim 4, wherein group R^1 represents glycyl or D-alanyl and group R^2 represents acetyl.

6. The compound according to any one of claims 1 to 5, wherein group R^1 represents $-NH_2$ and group R^2 represents glutamidyl or 2-aminopropionamidyl.

7. The compound according to any one of claims 1 to 6, wherein the toxin is selected from the group consisting of mycrocystin and nodularin congeners.

8. The compound according to any one of claims 1 to 7 which is a polyclonal, monoclonal or recombinant antibody or a functionally active derivative or fragment thereof.

9. A method for the preparation of the compound according to any one of claims 1 to 8 comprising the steps of

(a) preparing a compound containing a group represented by formula (I) as defined in any one of claims 1 to 7,

(b) coupling the compound of step (a) to a carrier.

10. The method according to claim 9, wherein the carrier is a polymeric substance.

AMENDED CLAIMS PER INTERNATIONAL PRELIMINARY EXAMINATION REPORT DATED 19 JULY 2001

11. The method according to claim 10, wherein the polymeric carrier is selected from the group consisting of polyethyleneglycol, polypeptides, proteins, polysaccharides or plastic supports.

12. The method according to claim 11, wherein the protein carrier is selected from bovine serum albumin, ovalbumin, cationised bovine serum albumin or horseradish peroxidase.

13. The method according to any one of claims 9 to 12 which further comprises the steps of
    (c) immunizing an animal with the conjugate obtained in step (b), and
    (d) isolating the animal's blood, blood serum and/or spleenocytes.

14. A diagnostic kit containing the compound according to any one of claims 1 to 8.

15. An affinity matrix containing the compound according to any one of claims 1 to 8 coupled to a polymeric resin.

16. Use of the compound according to any one of claims 1 to 8 for the detection of a compound containing the group represented by the formula (I).

17. A method for concentrating a compound containing the group represented by the formula (I) from a fluid or for substantially decreasing the amount of a compound containing the group represented by the formula (I) in a fluid com-

AMENDED CLAIMS PER INTERNATIONAL PRELIMINARY EXAMINATION REPORT DATED 19 JULY 2001

prising the steps of

(a) preparing the compound according to any one of claims 1 to 8,

(b) coupling the compound obtained in step (a) to a polymeric matrix, and

(c) contacting the fluid with the polymeric matrix obtained in step (b).

18. The method according to claim 17, wherein the fluid is hemodialysis water, drinking water or water derived from rivers, lakes and oceans.

16. Use of the compound according to claim 1 for the detection of a compound containing the group represented by the formula (I).

17. A method for concentrating a compound containing the group represented by the formula (I) from a fluid or for substantially decreasing the amount of a compound containing the group represented by the formula (I) in a fluid comprising the steps of

(a) preparing the compound according to claim 1,

(b) coupling the compound obtained in step (a) to a polymeric matrix, and

(c) contacting the fluid with the polymeric matrix obtained in step (b).

Please add the following new claims 19-28:

19. The compound according to claim 2, wherein:

the groups R^3 each represent methyl and group R^4 represents methoxy;

group R^1 represents aminoacyl and group R^2 represents (C_1-C_4)acyl;

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group R^1 represents glycyl or D-alanyl and group R^2 represents acetyl;

group R^1 represents $-NH_2$ and group R^2 represents glutamidyl or 2-aminopropionamidyl;

the toxin is selected from the group consisting of mycrocystin and nodularin congeners.

20. The compound according to claim 19 which is a polyclonal, monoclonal or recombinant antibody or a functionally active derivative or fragment thereof.

21. A method for the preparation of the compound according to claim 19 comprising the steps of

(a) preparing a compound containing a group represented by formula (I) as defined in claim 19,

(b) coupling the compound of step (a) to a carrier;

and wherein:

the carrier is a polymeric substance;

the polymeric carrier is selected from the group consisting of polyethyleneglycol, polypeptides, proteins, polysaccharides or plastic supports;

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the protein carrier is selected from bovine serum albumin, ovalbumin, cationised bovine serum albumin or horseradish peroxidase.

22. A method for the preparation of the compound according to claim 20 comprising the steps of

(a) preparing a compound containing a group represented by formula (I) as defined in claim 19,

(b) coupling the compound of step (a) to a carrier;

and wherein:

the carrier is a polymeric substance;

the polymeric carrier is selected from the group consisting of polyethyleneglycol, polypeptides, proteins, polysaccharides or plastic supports;

the protein carrier is selected from bovine serum albumin, ovalbumin, cationised bovine serum albumin or horseradish peroxidase.

23. The method according to claim 21 which further comprises the steps of

(c) immunizing an animal with the conjugate obtained in step (b), and

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(d) isolating the animal's blood, blood serum and/or spleenocytes.

24. The method according to claim 22 which further comprises the steps of

(c) immunizing an animal with the conjugate obtained in step (b), and

(d) isolating the animal's blood, blood serum and/or spleenocytes.

25. A diagnostic kit containing the compound according to claim 19.

26. An affinity matrix containing the compound according to claim 19 coupled to a polymeric resin.

27. Use of the compound according to claim 19 for the detection of a compound containing the group represented by the formula (I).

28. A method for concentrating a compound containing the group represented by the formula (I) from a fluid or for substantially decreasing the amount of a compound containing

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the group represented by the formula (I) in a fluid comprising the steps of

(a) preparing the compound according to claim 19,

(b) coupling the compound obtained in step (a) to a polymeric matrix, and

(c) contacting the fluid with the polymeric matrix obtained in step (b);

and wherein the fluid is hemodialysis water, drinking water or water derived from rivers, lakes and oceans.

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Attorney Docket No. MBP-010XX
Filed: Herewith
Group Art Unit:

REMARKS

This Preliminary Amendment puts the claims into proper form for examination. Note that claims 3, 4, 6-9 and 13-17 have been amended; new claims 19-28 have been added; and claims 1, 2, 5, 10-12 and 18 remain unchanged. Kindly calculate the filing fee based on the amended claims.

The Examiner is encouraged to telephone the undersigned attorney to discuss any matter which would expedite allowance of the present application.

Respectfully submitted,

DANIEL R. DIETRICH, ET AL.

By: _____
Charles L. Gagnebin III
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CLG/mc/268296-1
Enclosure

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Attorney Docket No. MBP-010XX
Filed: Herewith
Group Art Unit:

Red-lined claims for the Examiner's convenience:

3. The compound according to claim 1~~ or 2~~, wherein the groups R^3 each represent methyl and group R^4 represents methoxy.

4. The compound according to ~~any one of~~ claim~~s~~ 1~~ to 3~~, wherein group R^1 represents aminoacyl and group R^2 represents (C_1-C_4)acyl.

6. The compound according to ~~any one of~~ claim~~s~~ 1~~ to 5~~, wherein group R^1 represents $-NH_2$ and group R^2 represents glutamidyl or 2-aminopropionamidyl.

7. The compound according to ~~any one of~~ claim~~s~~ 1~~ to 6~~, wherein the toxin is selected from the group consisting of mycrocystin and nodularin congeners.

8. The compound according to ~~any one of~~ claim~~s~~ 1~~ to 7~~ which is a polyclonal, monoclonal or recombinant antibody or a functionally active derivative or fragment thereof.

9. A method for the preparation of the compound according to ~~any one of~~ claims 1~~ to 8~~ comprising the steps of

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(a) preparing a compound containing a group represented by formula (I) as defined in ~~any one of~~ claim~~s~~ 1~~ to 7~~,

(b) coupling the compound of step (a) to a carrier.

13. The method according to ~~any one of~~ claim~~s~~ 9~~ to 12~~ which further comprises the steps of

(c) immunizing an animal with the conjugate obtained in step (b), and

(d) isolating the animal's blood, blood serum and/or spleenocytes.

14. A diagnostic kit containing the compound according to ~~any one of~~ claim~~s~~ 1~~ to 8~~.

15. An affinity matrix containing the compound according to ~~any one of~~ claim~~s~~ 1~~ to 8~~ coupled to a polymeric resin.

16. Use of the compound according to ~~any one of~~ claim~~s~~ 1~~ to 8~~ for the detection of a compound containing the group represented by the formula (I).

17. A method for concentrating a compound containing the group represented by the formula (I) from a fluid or for

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substantially decreasing the amount of a compound containing the group represented by the formula (I) in a fluid comprising the steps of

(a) preparing the compound according to ~~any one of~~ claims ~~1 to 8~~,

(b) coupling the compound obtained in step (a) to a polymeric matrix, and

(c) contacting the fluid with the polymeric matrix obtained in step (b).

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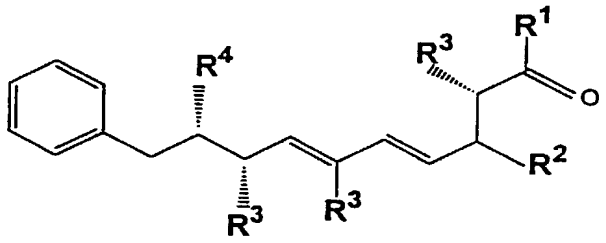
"Congener Independent Detection Of Microcystin And Nodularin Congeners"

Description

The present invention relates to a proteinaceous compound or functionally active derivative or part thereof having a binding site for a group represented by the following formula (I)



(I)

which is part of a group of toxins derived from various cyanobacteria, to a method for its production, to a diagnostic kits and to an affinity matrix (e.g. for use in immunoaffinity columns, online detection and purification devices) containing the proteinaceous compound as well as to methods for substantially decreasing the amount of a compound containing the group represented by formula (I) in fluids or for concentrating compounds, e.g. toxins, containing the group represented by formula (I) from fluids such as crude water samples, extracts of algae or other tissue samples, e.g. to determine toxin concentrations.

Due to increasing settlement, industrialisation and intensive agriculture wide spread problems of water pollution have arisen. This water pollution and the following eutrophication has led in many cases to the development of blooms of blue-green algae (i.e. cyanobacteria). The environmental factors which are responsible for the development of such blooms of cyanobacteria are up to now almost unknown. In general, blooms of cyanobacteria can be found in eutrophic bodies of water, e.g. under such conditions as relatively high nutrient levels (phosphate and nitrate), water temperatures of between 15 to 30°C and pH-values of between 6 and 9 or higher (Wicks et al., 1990).

A severe problem of the development of blooms of cyanobacteria is that cyanobacteria produce a broad variety of toxic substances. Accordingly, since the end of the last century there has been an increasing number of cases of intoxication and even deaths of humans, animals, especially birds and fishes, which could be demonstrated to be caused by the use of water which was contaminated with cyanobacteria after chlorination and filtration for medical purposes (cases of deaths in the dialysis centers of Caruaru, Brazil, 1996 and Evora, Portugal, 1995), by the consumption of contaminated drinking water or even of clumps of cyanobacteria themselves (Francis, 1878; Falconer et al., 1983; Carmichael, 1984; Beasley et al., 1989; Mahmod et al., 1988; Skolberg et al. 1984).

The toxin producing cyanobacteria can be subdivided into species which synthesize mostly hepatotoxic peptides such as *Microcystis* sp., *Nodularia* sp. and *Oszillatoria* sp., and other genus which produce mostly neurotoxic alkaloids such as *Anabaena* and *Aphanizomenon* (Carmichael et al., 1990). Studies of different strains of *M.aeruginosa* revealed that, depending on strain and habitat, the cyanobacteria produce different congeners and amounts of a toxin (Sivonen et al., 1992 a-c).

Cyanobacteria can secrete the intracellularly produced toxins into the surrounding water (Watanabe et al., 1992 a,b). Further studies showed that the microcystin congener microcystin-LR is photostable, however, it can be microbially degraded (Watanabe et al., 1992 a; Tsuji et al., 1994; Cousins et al., 1996). Under aerobic conditions and in culture media which were inoculated with bacteria, the half-lifetimes of microcystin-LR and -YR were more than 45 days (Watanabe et al., 1992 a). In contrast, half-lifetimes of less than 5 days were determined in seawater (Cousins et al., 1996). Under unfavorable conditions (i.e. cold temperatures and minimal presence of specific populations of microbes) microcystins may persist several days to even months and, therefore, may represent a potential danger for humans via the drinking water supply.

Accordingly, the increased incidence of gastroenteritis and liver carcinomas in humans has been attributed to the consumption of drinking water which was

contaminated with cyanobacterial hepatoxins (in particular microcystin-LR) in several studies, although a direct relation between chronic microcystin-LR exposure and the development of liver carcinomas has not yet been proven (Tisdale, 1931; Keleti et al., 1981; Billings, 1981). Clinical indications of microcystin toxicoses in mammals is characterized by weakness, anorexia, mucous pallor, muscle termor, forced expirations and death by hypovolemic shock which is caused by intrahepatic hemorrhagia and/or liver failure (Theiss et al., 1988; Jackson et al., 1984).

Mammals seem to take up microcystin orally, and the toxin reaches the liver with the blood via a highly specific transporter mechanism (organic anion carrier) (Eriksson et al, 1990; Hooser et al., 1990; Runnegar et al., 1991). One molecular mechanism of the serious effects of microcystin seems to be its binding to the catalytic subunit of proteinphosphatases 1 and 2A which leads to their inhibition (Eriksson et al., 1990; Yoshezawa et al., 1990; Matsushima et al., 1990; Honkanen et al., 1990; McKintosh et al., 1990; McKintosh et al., 1995; Runnegar et al., 1996). After accute intoxication of high microcystin concentrations, the inhibition of proteinphosphatases leads to hyperphosphorylation of intermediate filaments which, in turn, is followed by collapse of the cytoskeleton, loss of the cells' structure, extensive intrahepatic hemorrhage and necrosis of the hepatocytes (Eriksson et al., 1990; Falconer et al., 1981, 1992). Similar to other proteinphosphatase inhibitors (e.g. calyculin-A, okadaic acid), the chronic exposure of mice to microcystin-LR leads to promotion of liver tumors (Falconer, 1991; Nishiwaki-Matsushima et al., 1992).

Due to the high toxicity and carcinogenicity of hepatotoxic cyanobacteria toxins and the potential chronic exposure of organisms (humans as well as animals) to these toxins via the drinking water there is an urgent need to detect toxic blooms of cyanobacteria early and to decrease the concentration of cyanobacteria toxins in drinking water.

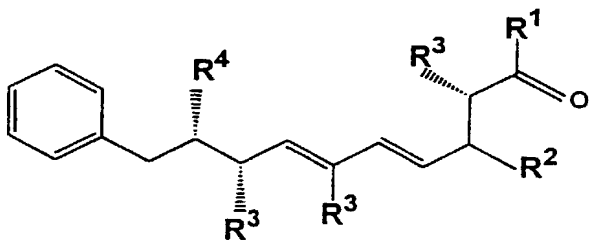
Since it has been difficult to analytically and routinely detect the different microcystin and nodularin congeners with the required sensitivity (Kenefick et al., 1993; Lawton et al., 1994), prior art studies have concentrated on the destruction

of the cyanobacteria toxins during the drinking water purification process. Mostly, continuous methods have been studied which can be carried out under routine conditions such as sand filtration, binding to activated carbon and destruction by chlorination (James et al., 1994). However, these studies revealed that neither sand filtration nor chlorination, UV-irradiation, treatment with hydrogen peroxide or potassium permanganate nor filtration via activated carbon could substantially remove the cyanobacteria toxins from drinking water. In this case a further problem seems to be the treatment of the raw water which is contaminated with cyanobacteria. The chlorination or the treatment of the cyanobacteria with copper sulfate leads to the release of the cyanobacteria toxins which are present in the cytosol without destroying the toxins to even the lowest degree. Also, the chlorination of sand filtered water is ineffective. Only the filtration via activated carbon seems to be appropriate to remove a considerable amount (about 60% to 80%) of the toxins. However, this purification performance was only reached for a limited period of time due to a relatively quick saturation of the activated carbon particles. Therefore, after about 10,000 bed volumes (1 bed volume = volume of the activated carbon) the filters became leaky.

Therefore, the technical problem underlying the present invention is to provide a novel system for the reliable detection as well as the removal of all kinds of hepatotoxic cyanobacteria toxins such as microcystin and nodularin congeners, particularly in and from, drinking water and other sources.

The solution to the above problem is provided by the embodiments of the present invention as characterized in the claims.

In particular, the present invention relates to a proteinaceous compound or functionally active derivative or part thereof having a binding site for a group represented by the following formula (I)



(I)

which is part of a toxin derived from a cyanobacterium, wherein
group R^1 represents a halogen atom, preferably Br, $-OSO_3$, -OR' or $-NR'_2$
group R^2 represents hydrogen, (C_1-C_4)alkyl, (C_1-C_4)alkoxy, (C_1-C_4)acyl, (C_1-C_4)aminoacyl or (C_1-C_4)carboxaminoacyl,
or the groups R^1 and R^2 are connected to each other to form a cyclic compound,
the groups R^3 which may be the same or different are each independently selected from the group consisting of hydrogen and (C_1-C_4)alkyl,
group R^4 represents (C_1-C_4)alkoxy,
and wherein the phenyl group may be substituted or unsubstituted.

The term "proteinaceous compound or functionally active derivative or part thereof" means a compound which is capable of binding the above-described group of formula (I) and substantially consists of one or more polypeptides. The functionally active form of the proteinaceous compound according to the present invention may be a monomeric or a homo- or heterodimeric, -trimeric, -tetrameric or other oligomeric form.

The term "binding site" for the group as defined above means a three-dimensional arrangement of atoms of the above proteinaceous compound which is able to specifically interact with the group of formula (I) as defined above. The specific interaction may be any kind of chemical and/or physical interaction and comprises covalent binding, electrostatic interactions, hydrogen bonding, Van-der-Waals- as well as hydrophobic interactions.

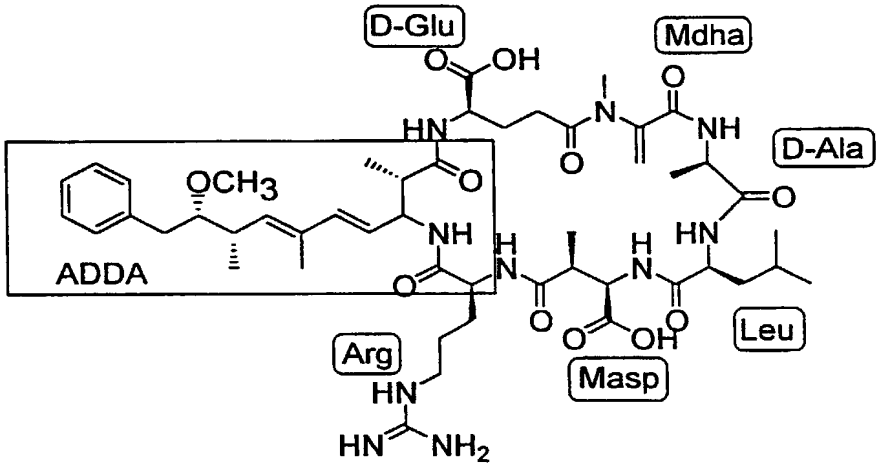
Preferably, the group R' in the formula (I) represents independently from each other hydrogen, substituted or unsubstituted (C_1-C_4)alkyl or (C_1-C_4)acyl, when bound to nitrogen. According to a further preferred embodiment of the proteinaceous compound as defined above, the groups R^3 in the above formula (I) each represent methyl and group R^4 represents methoxy.

According to a further preferred embodiment of the proteinaceous compound of the present invention, group R^1 represents aminoacyl and group R^2 represents (C_1-C_4)acyl, or group R^1 represents glycyl or D-alanyl, respectively, and group R^2

represents acetyl, or group R^1 represents $-NH_2$ and group R^2 represents glutamidyl or 2-aminoproprionamidyl, respectively.

Preferably, the group represented by the above formula (I) is part of a toxin selected from the group consisting of microcystin and nodularin congeners. Microcystin (MC) and nodularin congeners are hereinafter referred to as microcystin-XY and nodularin-XY.

The chemical structures of *M. aeruginosa* and *Nodularia* sp.-hepatotoxins (i.e. microcystin-XY and nodularin-XY) are described in several prior art studies (Botes et al., 1982 a, d, 1994, 1985; Rinehard et al., 1988). Microcystin-XY and nodularin-XY are cyclic peptides consisting of seven or five, respectively, amino acids. The following formula represents microcystin-LR.



Nodularin-XY and microcystin-XY share the same specific characteristic amino acid (ADDA). The basic structure of microcystin-XY congeners consists of five non-variable amino acids: β-methylasparaginic acid, alanine, N-methyl-dehydroalanine, glutamate, and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienic acid (ADDA). The differences between individual microcystin congeners are based on the two variable L-amino acids which are, for

example, L-arginine and L-leucine in microcystin-LR and two times L-arginine in microcystin-RR, respectively. Normally, cyanobacteria produce a mixture of different forms of the toxins. The isolation of microcystin-XY from natural blooms of blue-green-algae resulted in up to six different microcystin congeners, and toxin concentrations up to 10 mg per g of dry mass of algae were determined (Wicks et al., 1990; Tsuji et al., 1994; Tencallar et al., 1994, 1995).

An especially preferred example of the proteinaceous compound according to the present invention is a polyclonal, monoclonal or recombinant antibody or a functionally active derivative or fragment thereof. The recombinant antibody may be produced by the translation and expression of any part of the genes coding for polyclonal or monoclonal antibodies and/or selection by screening of a phage display library using the group represent by the above formula (I).

The proteinaceous compound according to the present invention, e.g. a polyclonal, monoclonal or recombinant antibody or functionally active derivative or fragment thereof, has the advantage to be capable of binding to all congeners of the cyanobacterial hepatotoxins, e.g. microcystin and nodularin congeners which contain as a part of their structure the ADDA moiety.

In contrast to the proteinaceous compound of the present invention, the commercially available antibodies or ELISA kits, respectivley, are only capable of recognizing a very limited number of microcystin congeners. This means that the toxicity of blooms of cyanobacteria can be massively underdetermined by the use of the antibodies or kits, respectively, known so far.

A further embodiment of the present invention relates to a method for the preparation of the proteinaceous compound as defined above, comprising the steps of

a) preparing a compound containing a group represented by the formula (I) as defined above and

(b) coupling the compound of step (a) to a carrier.

The „carrier" is not particularly limited to a specific embodiment and may be, e.g. any polymeric substance. For example, carriers which are suitable for the above method may be selected from the group consisting of polyethyleneglycol, proteins, polypeptides, polysaccharides and solid phase supports such as plastic supports. Preferably, the protein carrier is selected from bovine serum albumin (BSA), ovalbumin (OVA) cationised bovine serum albumin (cBSA), and horseradish peroxidase (HRP).

In another preferred embodiment of the present invention, the above method further comprises the steps of

(c) immunizing an animal with the conjugate obtained in step (b) above and

(d) isolating the animal's blood, blood serum and/or spleenocytes.

In a further preferred embodiment, the above method further comprises the steps of preparing antisera from the animal's blood serum obtained in the above step (d) for the preparation of polyclonal antibodies. According to another preferred embodiment, the method of the present invention further comprises the steps of preparing monoclonal antibody-producing hybridoma cells from the animal's spleenocytes obtained in the above step (d). Yet another preferred embodiment of the above-defined method comprises the further steps of preparing recombinant antibodies including the isolation of the genetic material (DNA) from cells present in the animal's blood or from antibody-producing hybridoma cells.

A further embodiment of the present invention relates to a diagnostic kit containing the proteinaceous compound as defined above.

Another embodiment of the present invention relates to an affinity matrix containing the proteinaceous compound as defined above coupled to a polymeric resin.

The proteinaceous compound according to the present invention, e.g. a polyclonal, monoclonal or recombinant antibody or a functionally active derivative or fragment thereof as defined above, is particularly useful for the detection of a

compound containing the group represented by the above formula (I), for concentrating the toxins from crude extracts prior to analysis to determine toxin concentrations as well as to substantially decrease the amount of a compound containing the group represented by the formula (I) in a fluid, pharmaceutical or food preparation.

Therefore, a further embodiment of the present invention relates to a method for concentrating a compound containing the group represented by the formula (I), e.g. a toxin, from a fluid such as crude water samples, extracts of algae or other tissue samples, or for substantially decreasing the amount of a compound containing the group represented by the formula (I) in a fluid, e.g. water such as hemodialysis water, drinking water or water derived from rivers, lakes and oceans, comprising the steps of

(a) preparing the proteinaceous compound as defined above,

(b) coupling the compound obtained in step (a) to a polymeric matrix, and

(c) contacting the fluid with the polymeric matrix obtained in step (b).

Furthermore, the above method may also be applied to the cleaning of any other sources of cyanobacteria toxins such as, for example, food stuffs.

The Figures show:

Fig. 1 is a diagram showing a flow chart for the strategy of preparation of an anti-ADDA antibody according to the present invention.

Fig. 2 is a diagram showing preferred strategies for the coupling of an ADDA-hapten to a protein.

Fig. 3 shows several ADDA-derivatives which were synthesized for the production of the antibody useful for congener independent detection of microcystin and nodularin congeners.

Fig. 4 is a diagram showing the general principle of the indirect competitive microcystin enzyme-linked immunosorbent assay (MC-ELISA).

Fig. 5 is a diagram showing the crossreactivity with respect to different microcystin congeners (MC-LR, -RR and -YR) and nodularin of the anti-ADDA antibody (ADDA-824new, i.e. 26/06/00) raised in sheep which is directed against ADDA-HG coupled to ovalbumin.

Fig. 6 is a diagram showing the direct ELISA method and its sensitivity for detection of MC-YR. The anti-ADDA antibody (ADDA-825-$^{bleed,14/12/98}$) was raised in sheep and directed against ADDA-HG coupled to ovalbumin.

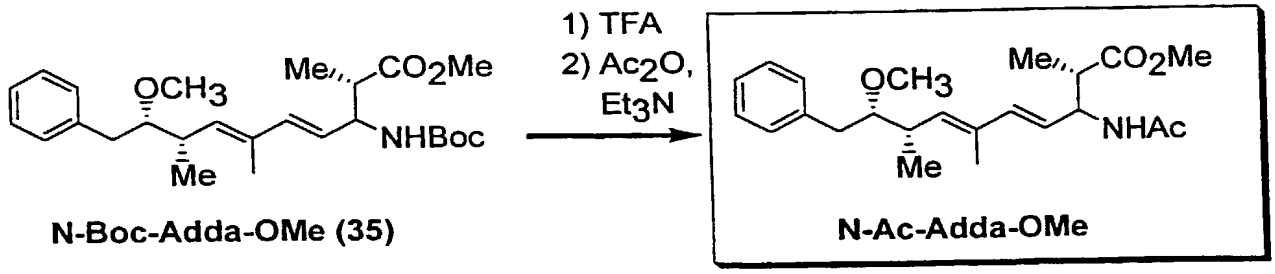
Fig. 7 is a diagram showing the indirect ELISA method using a monoclonal antibody and ist sensitivity for detection of MC-YR. The anti-ADDA antibody (ADDA-3G10B10) was raised in mice and directed against ADDA-HG coupled to ovalbumin.

The present invention is further illustrated by the following non-limiting examples.

EXAMPLE

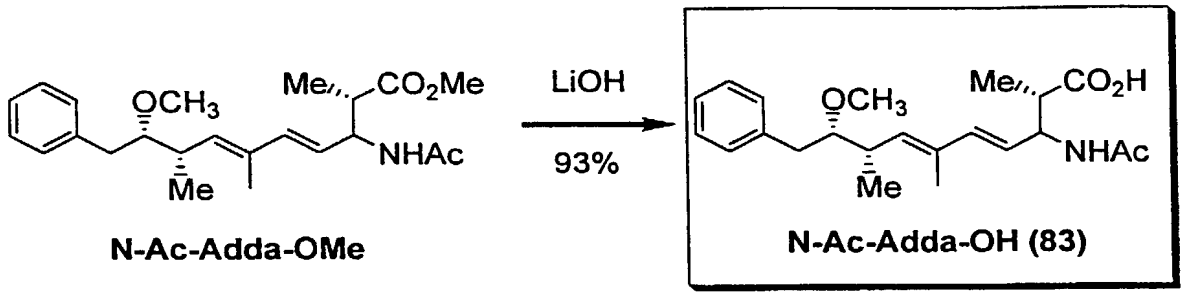
ADDA hapten synthesis

The starting material N-Boc-ADDA-Me (**35**) was prepared by the published route: Humphrey, J. M.; Aggen, J.; Chamberlin, A. R. *J. Am Chem. Soc.* **1996**, *118*, 11759-11770. "Synthesis of the Serine-threonine Phosphatase Inhibitor Microcystin LA."



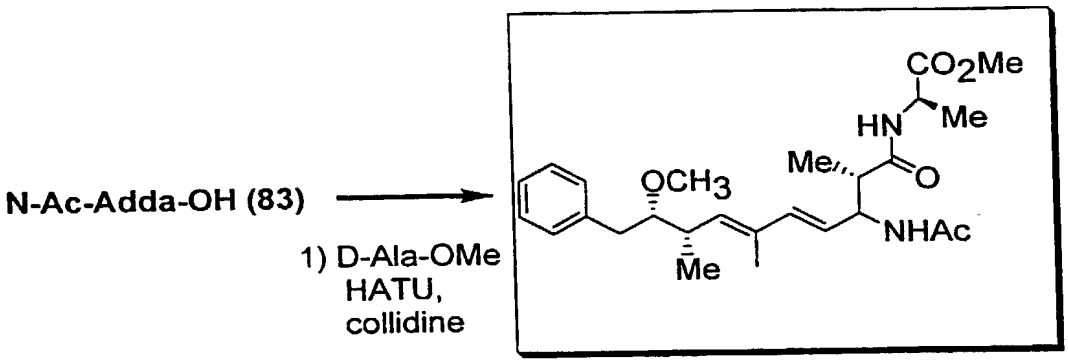
N-Boc-Adda-OMe (35) **N-Ac-Adda-OMe**

N-Ac-ADDA-OMe. To 31 mg (0.70 mmol) of **Boc-ADDA-OMe** in a flask was added 2 ml of TFA. After one hour the TFA was removed under vacuum, and the residue was concentrated three times from toluene to remove the TFA. The resulting oil was dissolved in 2.5 ml of freshly distilled CH_2Cl_2, and this was cooled to 0° C. 28 mg (0.28 mmol) of anhydrous triethylamine was added to the solution, followed by 0.141 g (1.39 mmol) of freshly distilled acetic anhydride. One hour later 5 ml of saturated NH_4Cl was added, and the mixture was stirred for 20 minutes at 0° C. The mixture was partitioned between water and EtOAc, and the phases were separated. The aqueous phase was extracted twice with EtOAc. The combined organic phases were washed once each with 50% saturated NH_4Cl, 50% saturated $NaHCO_3$, and brine, dried over $MgSO_4$, filtered, and concentrated under vacuum to give a white solid. The solid was purified via flash chromatography (1/1 EtOAc/hexanes) to give 25 mg (93%) of a white solid: R_f 0.23 (40:60 EtOAc:hexanes); IR (thin film) 3330, 2919, 1731, 1654, 1454 cm^{-1}; ^1H-NMR (500 MHz, $CDCl_3$) ‰ 0.99 (d, J = 6.5, 3H), 1.20 (d, J = 7, 3H), 1.57 (s, 3H), 2.02 (s, 3H), 2.58 (ddq, J = 6, 6.5, 10 Hz, 1H), 2.67 (dd J = 7.5, 14 Hz, 1H), 2.78 (obscured mult., 3H), 2.79 (dd, J = 5, 13.5 Hz, 1H), 3.17 (ddd, J = 5, 6, 7 Hz, 1H), 3.21 (s, 3H), 3.65 (s, 3H), 4.71 (ddd, J = 4.5,5,5.5 Hz, 1H), 5.37 (d, J = 9.5 Hz, 1H), 5.42 (dd, J = 15.5, 6.5 Hz, 1H), 6.18 (d, J = 15.5 Hz, 1H), 6.40 (d, J = 9 Hz, 1H), 7.25-7.15 (m, 5H); ^{13}C NMR (125 MHz, $CDCl_3$) ‰175.8, 169.5, 139.5, 136.6, 136.3, 132.4, 129.5, 128.2, 126.0, 124.9, 87.1, 58.6,_ 3.0, 51.7, 43.6, 38.4, 36.8, 23.5, 16.2, 14.9, 12.7; HRMS calculated for $C_{23}H_{34}NO_4$ $(M+H)^+$: 388.2488. Found: 388.2505.

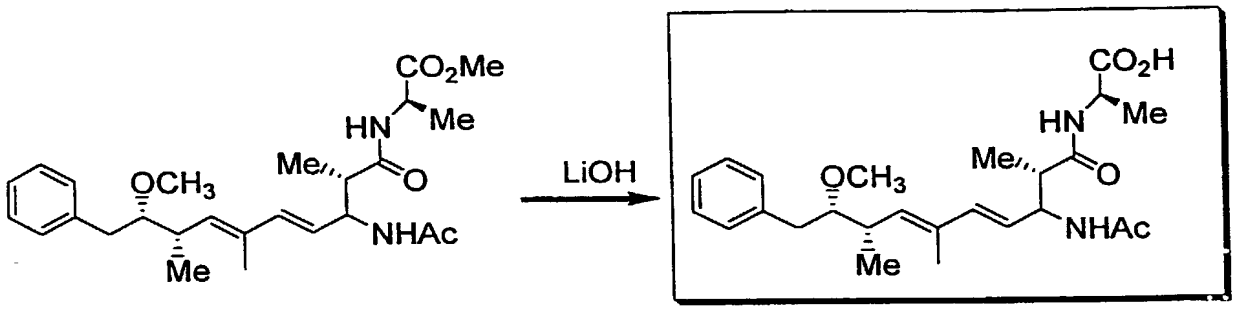


N-Ac-Adda-OMe **N-Ac-Adda-OH (83)**

N-Ac-ADDA-OH. To 22 mg (0.057 mmol) of the protected ADDA-derivative in 2 ml THF was added 0.57 ml (0.57 mmol) of 1 M LiOH. After 22 hours the mixture had clarified, and it was partitioned between hexanes and water. The phases were separated, and the aqueous phase was washed once with hexanes. The combined organic phases were back-extracted three times with water. The combined aqueous phases were acidified with 1 M $NaHSO_4$, and extracted three times with CH_2Cl_2. The combined CH_2Cl_2 phases were washed once with brine, filtered through cotton, and concentrated to give 23 mg of **83** as an oil that was taken on without purification: R_f 0.34 (1:49:50 HOAc:EtOAc:hexanes); IR (thin film) 3295 br, 2923, 1713, 1640 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) ‰0.99 (d, J = 6.5, 3H), 1.25 (d, J = 7, 3H), 1.58 (s, 3H), 2.02 (s, 3H), 2.57 (ddq, J = 6.5, 6.5, 9.5 Hz, 1H), 2.65 (dd J = 7.5, 14 Hz, 1H), 2.76 (par.obsc. m, 3H), 2.77 (dd, J = 5, 13 Hz, 1H), 3.17 (ddd, J = 5, 6.5, 6.5 Hz, 1H), 3.21 (s, 3H), 4.71 (ddd, J = 5, 6, 10 Hz, 1H), 5.37 (d, J = 9.5 Hz, 1H), 5.45 (dd, J = 15.5, 6.5 Hz, 1H), 6.18 (d, J = 15.5 Hz, 1H), 6.37 (d, J = 9.5 Hz, 1H), 7.25-7.15 (m, 5H); HRMS calculated for $C_{22}H_{32}NO_4$ $(M+H)^+$: 374.2331, Found: 374.2325.



N-Ac-ADDA-D-Ala-OMe. To 17 mg (0.12 mmol) of D-Ala-OMe hydrochloride and 14 mg (0.036 mmol) of HATU in a flask was added 9 mg (0.024 mmol) of **83** in 0.6 ml DMF. The resulting solution was cooled to 0° C, and 41 mg (0.34 mmol) of collidine was added. The solution was stirred at 0° C for 2 hours, followed by warming to room temperature and stirring overnight. The mixture was partitioned between water and EtOAc, and the phases were separated. The aqueous phase was extracted once with EtOAc. The combined organic phases were washed once each with sat. $NaHCO_3$, water, 1 M $NaHSO_4$, water, and brine, dried over $MgSO_4$, filtered, and concentrated under vacuum to an off-white solid. Chromatography (80:20 EtOAc:hexanes) gave 8 mg (73%) of a white solid: R_f 0.17 (60:40 EtOAc:hexanes); IR (thin film) 3284, 3067, 2923, 1743, 1650, 1542 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) ‰ 0.99 (d, J = 6.5, 3H), 1.23 (d, J = 7, 3H), 1.35 (d, J = 7 Hz, 3H), 1.58 (s, 3H), 2.04 (s, 3H), 2.52 (dq, J = 4, 7 Hz, 1H), 2.59 (ddq, J = 6.5, 7, 9.5 Hz, 1H), 2.68 (dd J = 7.5, 14 Hz, 1H), 2.81 (dd, J = 4.5, 14 Hz, 1H), 3.19 (ddd, J = 5, 7, 7 Hz, 1H), 3.22 (s, 3H), 3.75 (s, 3H), 4.55 (dq, J = 7, 7 Hz, 1H), 4.62 (m, 1H), 5.39 (d, J = 9.5 Hz, 1H), 5.45 (dd, J = 15.5, 6.5 Hz, 1H), 6.18 (d, J = 15.5 Hz, 1H), 6.23 (d, J = 7 Hz, 1H), 7.05 (d, J = 9 Hz, 1H), 7.27-7.17 (m, 5H); ^{13}C NMR (125 MHz, $CDCl_3$) ‰ 12.7, 15.4, 16.2, 18.4, 23.5, 36.7, 38.2, 44.4, 47.9, 52.6, 53.7, 58.6, 86.9, 125.2, 125.9, 128.2, 129.4, 132.2, 136.2, 139.4, 169.9, 173.2, 174.6; HRMS calculated for $C_{26}H_{39}N_2O_5$ $(M+H)^+$: 459.2859, Found: 459.2869.

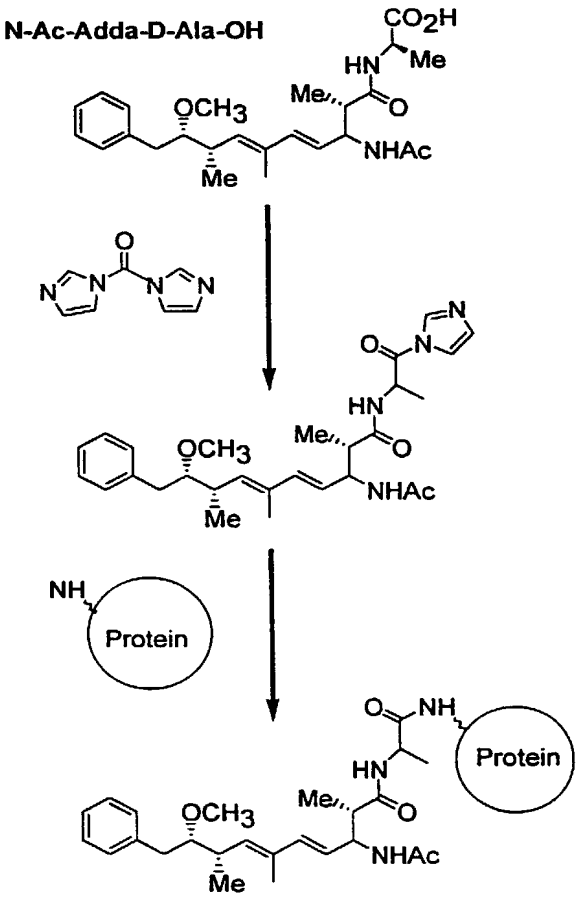


N-Ac-ADDA-D-Ala-OH. To 5 mg (0.011 mmol) of N-Ac-ADDA-D-Ala-OMe in 1.75 ml of THF was added 0.10 ml (0.10 mmol) of 1 M LiOH. After 50 minutes, the mixture was partitoned between ether and water, and the phases were separated. The aqueous phase was washed once with ether. The combined etheral phases were back-extracted three times with water, and the combined aqueous phases were acidified to pH = 3 with saturated citric acid. The aqueous phases were then extracted twice with EtOAc, and the combined EtOAc phases were washed twice with water, once with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting solid was purified by preparative reversed-phase HPLC, retention time of product = 15.7 minutes (70 MeOH / 30 0.2% aq. TFA), to give 4 mg (85%) of the title compound as a white solid: R_f 0.36 (1 HOAc / 10 MeOH / 89 CH_2Cl_2); IR (thin film) 3288 br, 2937, 1720, 1658, 1632 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) ‰ 0.94 (d, J = 7.0 Hz, 3H), 0.96 (d, J = 7.0 Hz, 3H), 1.19 (d, J = 7.0 Hz, 3H), 1.52 (s, 3H), 1.82 (s, 3H), 2.63 (dd, J = 7.0, 14.0 Hz, 1H), 2.73 (dd, J = 5.0, 14.0 Hz, 1H), 3.16 (s, 3H), 3.22 (ddd, J = 5.5, 5.5, 6.5 Hz, 1H), 4.19 (dq, J = 7.0, 7.5 Hz, 1H), 4.40 (m, 1H), 5.38 (d, J = 10.0 Hz, 1H), 5.44 (dd, J = 6.5, 16.0 Hz, 1H), 6.05 (d, J = 16.0 Hz, 1H), 7.17 (d, J = 7.5 Hz, 3H), 7.25 (t, J = 7.5 Hz, 2H), 7.60 (d, J = 9.0 Hz, 1H), 8.01 (d, J = 7.0 Hz, 1H); FAB MS calculated for $C_{25}H_{37}N_2O_5$ $(M+H)^+$: 445.2702. Found: 445.2695.

Coupling of hapten to proteins

Preparation of BSA–, cBSA–, and OVA–*N*-AcADDA-AlaOH.

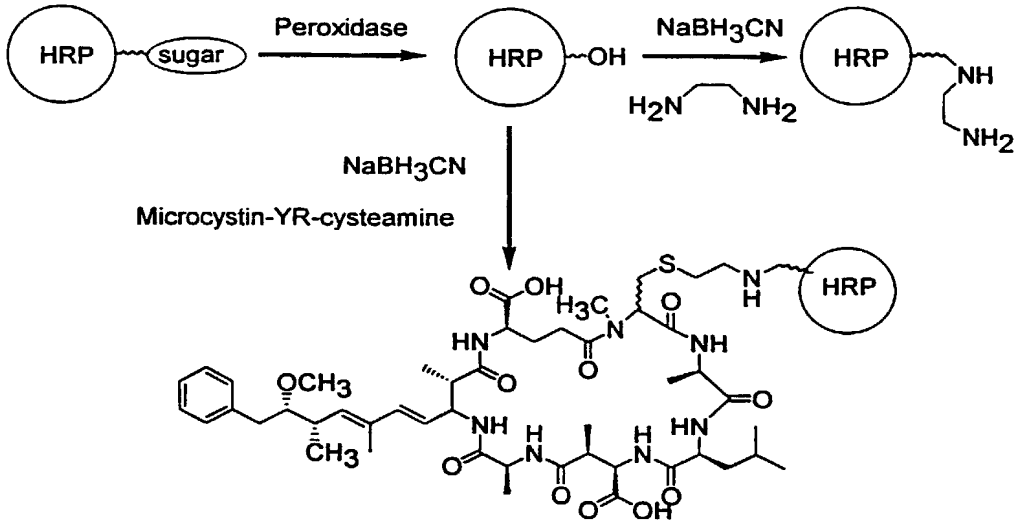
BSA (10.6 mg), cationised BSA (cBSA) (10.0 mg), and OVA (8.3 mg) were each dissolved in PBS (1000 µl). Carbonyldiimidazole (19.81 mg, 0.12 mmol) was dissolved in dry DMF (500 µl), and a portion of the solution (100 µl) was added to *N*-acetyl-ADDA-D-Ala-OH (1.0 mg, 2.2 µmol) and allowed to stand for 90 min. DMF was added (BSA, 260 µl; cBSA, 260 µl; OVA, 280 µl) to the protein solutions just prior to addition of the activated ADDA-derivative. The solution of the activated ADDA-derivative (40 µl each to the BSA and cBSA, 20 µl to the OVA) was then added to the protein solutions, and the reaction was allowed to proceed at 4 °C for about 16 h. The resulting conjugates were repeatedly diluted and then concentrated by ultrafiltration (Filtron centrifugal ultrafiltration tubes, 10K cutoff) until the calculated dilution of unretained low molecular weight compounds was > 10^6.



N-Ac-Adda-D-Ala-Protein Conjugate

Preparation of HRP–MC-YR and aminoHRP.

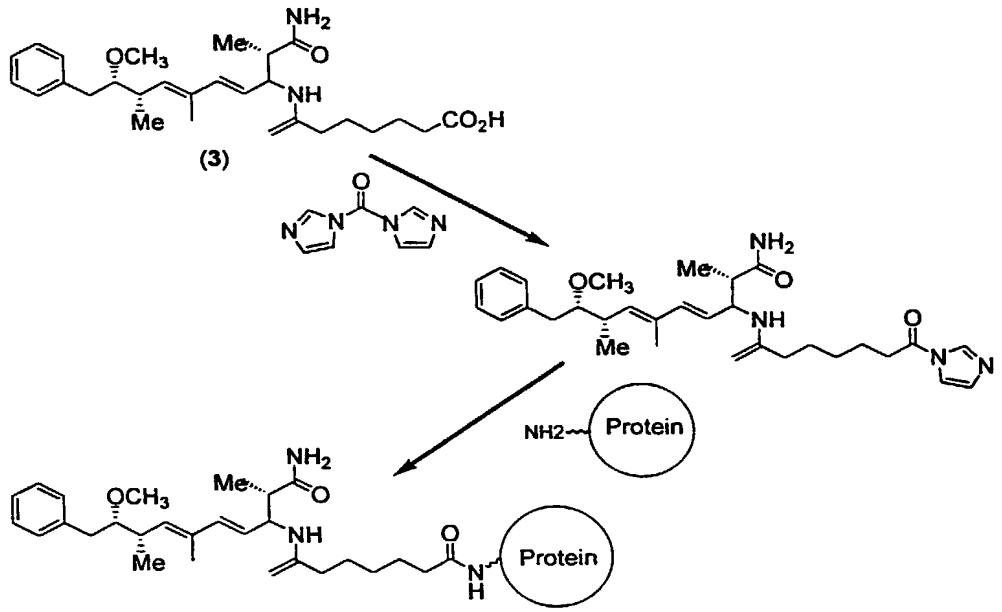
Horse radish peroxidase (HRP) was oxidized by the method of Hermanson. HRP (19.73 mg, Boehringer) was dissolved in PBS and cooled to 4 °C. $NaIO_4$ (36.7 mg) was dissolved in water (2 ml), and 100 µl of this was added to the HRP solution, which rapidly became green. The reaction was held at 4°C in the dark for 20 min, then the HRP was separated from low molecular weight material by elution with PBS through a desalting column (Bio-Rad Econo-Pac 10DG).



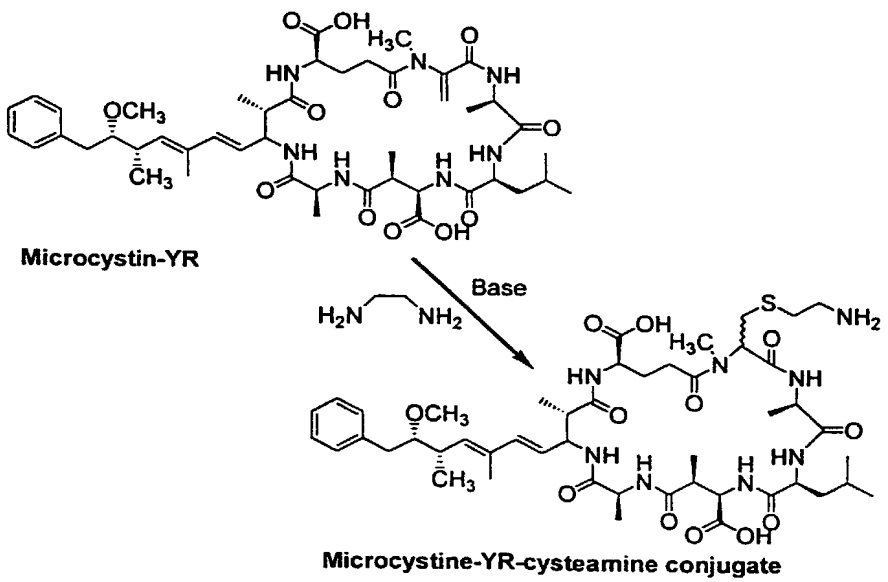
To half of the oxidized HRP MC-YR-cysteamine (51 µg, see below) was added in MeOH (50 µl). To the other half diaminoethane hydrochloride (500 mg) was added in PBS (500 µl). $NaBH_3CN$ (16.4 mg) was dissolved in PBS (500 µl), and 100 µl of this was added to each HRP reaction (which immediately became crimson). After standing at 4 °C in the dark for about 16 h, the reactions were quenched by addition of diethanolamine in PBS (50 µl of 300 µl of diethanolamine in 5 ml PBS) and allowed to stand at 4°C in the dark for 2 h. The HRP solutions were then purified by passing through desalting columns (as above). The diaminethane conjugate (henceforth referred to as aminoHRP) and MC-YR conjugates were further purified by ultrafiltration to > 10^4 dilution (as above).

Preparation of HRP–, aminoHRP–, and OVA–ADDA-HG.

HRP, aminoHRP, and OVA were each dissolved in PBS (1 ml). To Me-ADDA-HG (0.67 mg) was added CDI (1.16 mg) in dry DMF (100 µl), and the reaction proceeded at ambient temperature 1.5 h whereupon dry DMF (150 µl) was added. A portion of this solution was added to the solutions of the proteins (50 µl to aminoHRP, 100 µl to HRP and OVA). DMSO (200 µl) was then added to the HRP and OVA reactions to assist in solubilising the reactants, and the three reactions were maintained at 4 °C in the dark for ca 16 h. The conjugates were then purified on the desalting column and then further purified by repeated ultracentrifugation to > 3×10^4 dilution (as above).



Preparation of MC-YR–cysteamine conjugate



The method is based on those of Kondo et al. (1992) and Sherlock et al. (1998). Cysteamine (15.6 mg) was dissolved in water (500 µl), and MC-YR (500 µg) was dissolved in 5% K_2CO_3 (500 µl). The cysteamine solution (50 µl, followed by 100 µl at 30 min) was added to the MC-YR solution in portions. After about 2 h the reaction was acidified to pH 3 to 4 and applied to a reverse-phase flash column (4 × 1 cm). The column was eluted successively with water (10 ml), 10% MeOH (10 ml), 20% MeOH (10 ml), 30% MeOH (10 ml), 50% MeOH (2 × 10 ml), 70% MeOH (2 × 10 ml), and MeOH (3 × 10 ml). HPLC analysis indicated the product to be in the 50% MeOH and the first of the 70% MeOH fractions. These fractions were combined and the solvent removed *in vacuo* to yield MC-YR-cysteamine as a colourless solid (204 µg). ESI-MS *m/z* 1121.9 ($M-H^-$); ^1H, COSY and HMBC NMR spectra were consistent with the desired product.

Immunization of sheep and mice with ADDA-protein conjugates

Nine sheep and nine mice were immunised with the above BSA-ADDA-, cBSA-ADDA- and OVA-ADDA-conjugates (three animals for each conjugate). One mg of each conjugate in a volume of 1 ml phosphate buffer saline was added to Freund's complete adjuvants in case of the primary injection and homogenised to form an emulsion, and Freunds incomplete adjuvants in the case of booster injections. The animals received a minimum of three boosts in case of sheep, and six boosts in case of mice at approximately 4-week intervals.

ELISA

Indirect ELISA using polyclonal antibody #824

ELISA plates (NUNC MaxiSorp 1 #439454, Denmark) were coated with OVA-N-acetyl-D-alanyl-ADDA conjugate (OVA-ADDA-HG3/99') in 0.05 M sodium bicarbonate buffer pH 9.6 (75 µl, 2.5 µg/ml) overnight at 22 °C (RT). After a wash with PBS, additional binding sites were blocked by incubation with OVA (1/% w/v, 300 µl, 1 h, 20-25°C). Plates were washed two times with PBS and used immediately or stored at 4 °C for up to 7 days. In the assay, sample or standard (50 µl) was added to the wells together with antiserum (50 µl) at the appropriate

dilution (e.g. sheep serum #824$^{26/6/00}$ at 1/200 000; cf. Fig. 5). After incubation at 20-25 °C for 2 h, wells were washed twice with PBS + 0.05% Tween® 20 (PBST) and twice with PBS. Secondary antibody, horse radish peroxitase conjugated anti-species antibody, e.g. ICN/Cappel Anti-sheep-HRP (100µl, 1/6000), was then added to the wells and incubated for 2 h. Thereafter, wells were aspirated, washed twice with PBST and twice with PBS. TMB substrate solution, prepared by adding 110 µl TMB stock (10 mg/ml in DMSO) to 11 ml of sodium acetate buffer (0.1 M, pH 5.5) containing 0.005% H_2O_2, was then added and incubated for 15 minutes. The reaction was stopped by addition of H_2SO_4 (50 µl, 2 M), and the absorbance at 450 nm was determined with a microplate spectrophotometer. Standards and samples were prepared for ELISA by dilution in the following diluents: (i) methanol in PBS to a maximum methanol concentration of 10% (v/v); (ii) PBS; (iii) lake water (Lake Constance, Germany); (iv) tap water; (v) river water, Waikato River, New Zealand. All samples were analysed in at least duplicate, and over a range of dilutions.

ELISA method using antibody ADDA-#824$^{26/6/00}$ in detail

The principle of the indirect competitive microcystin ELISA (MC-ELISA) employed is shown in Fig. 4. This method comprises the following steps:

1. Prepare antigen (OVA-ADDA-HG$^{3/99}$) in bicarbonate buffer, pH 9.6 at 2.5 µg/ml (5 ml +/ plate).
2. Coat antigen onto microtitre plate at 75 µl/well, tap to mix and cover, incubate overnight at room temperature (RT, 22°C).
3. Wash two times in PBS, aspirate.
4. Block plate with 1% OVA (no. A-5503 from Sigma) (300 µl for 1 hour at RT (22 °C).
5. Wash two times in PBS, aspirate and use immediately or add 2-300 µl PBS for storage.

   The plates can be stored at this stage in PBS (at 4 °C) for up to 7 days.
6. Add 50 µl sample, or standard, in PBS;

   and 50 µl of antibody **ADDA- #824$^{26/6/00}$** (developed in sheep) at 1/200 000 dilution in OVA-blocker and incubate for 2 hours at RT (22 °C).

Standard curve: Primary 5000 ng/ml, then nine serial 1:8 dilutions (1 + 7) in 10%MeOH/PBS.

7. Wash two times in PBS/Tween, two times in PBS.
8. Add 100 µl of secondary antibody conjugate diluted in OVA (peroxidase-conjugated rabbit-anti-sheep IgG (ICN #55814) at a final dilution of 1/6000 and incubate for 2 hours at RT (22 °C).
9. Wash two times in PBST, two times in PBS, aspirate.
10. Turn on plate reader – needs a 15 minute warm up before reading at step 13.
11. Add 100 µl of substrate. Incubate for 15 minutes at RT (22 °C) until colour develops.
12. Add 50 µl stop solution (2M H_2SO_4).
13. Read absorbance at 450 nm. Note that the absorbance at 655 nm can be measured prior to adding the stop solution if required.

Direct ELISA using polyclonal antibody #825

ELISA plates (NUNC Maxisorp 1 # 439454, Denmark) were coated with the appropriate antiserum (#825$^{14/12/98}$) in 0.05 M sodium bicarbonate buffer pH 9.6 (50 µl, 1/20 000) overnight at 20 °C. After a 2 x PBS wash, additional binding sites were blocked by incubation with BSA (1% w/v, 300 µl, 1 h, 20-25°C). Plates were washed two times with PBS and used immediately or stored at 4 °C for up to 7 days. In the assay, sample or standard (50 µl) was added to the wells together with the appropriate hapten-enzyme conjugate (50 µl, NH_2-ADDA-HRP$^{3/99}$, 200 ng/ml). After incubation at 20-25 °C for 3 hours, wells were washed twice with PBST and twice with PBS. TMB substrate solution, prepared by adding 110 µl TMB stock (10 mg/ml DMSO) to 11 ml sodium acetate buffer (0.1 M pH 5.5) containing 0.005% H_2O_2, was then added, followed by incubation for 15 minutes. The reaction was stopped by addition of H_2SO_4 (50 µl, 2 M), and the absorbance was determined with a microplate spectrophotometer at a wavelength of 450 nm. Standards and samples were prepared for ELISA by dilution in the following diluents: (i) methanol in PBS to a maximum methanol concentration of 10% (w/v); (ii) PBS; (iii) lake water (Lake Constance, Germany); (iv) tap water; (v) river water (Waikato River, New Zealand). All samples were analysed at least in duplicate

and over a range of dilutions.

Direct ELISA method in detail (example 99153005).

1. Prepare antiserum (#825, developed in sheep) in bicarbonate buffer pH 9.6 at 1/20 000 (5 ml\plate). Coat microtitre plate with 50 µl antiserum per well, tap to mix and cover, incubate overnight at room temperature (RT, 22 °C).
2. Wash 2 x PBS, aspirate.
3. Block plate with 1% BSA (300 µl for 1 h at RT (22 °C)).
4. Wash 2 x PBS, aspirate and use or add 200-300 µl PBS for storage. The plates can be stored at this stage in PBS (at 4 °C) for up to 7 days.
5. Add 50 µl sample, or standard in PBS, and 50 µl of hapten-enzyme conjugate (NH_2-ADDA-HRP) 200 ng/ml in BSA-blocker and incubate at room temperature for 3 hours at RT (22 °C).
   Standard curve primary 2000 ng/ml, then 9 serial 1:6 dilutions in PBS.
6. Wash 2 x PBST, 2 x PBS. Aspirate.
7. Turn on plate reader – needs a 15 minute warm up before reading at step 10.
8. Add 100 µl of substrate. Incubate at RT (22 °C) for 15 minutes.
9. Add 50 µl stop solution (2 M H_2SO_4).
10. Read absorbance at 450 nm. Note that the absorbance at 655 nm can be measured prior to adding the stop solution if required.

Results of the above-described test are illustrated in Fig. 6.

Indirect ELISA using monoclonal antibody #3G10B10 (assay 9910n001)

ELISA plates (NUNC MaxiSorp 1 #439454, Denmark) were coated with OVA-ADDA-HG conjugate in 0.05 M sodium bicarbonate buffer pH 9.6 (50 µl, 2.5 µg/ml) overnight at 20 °C. After a wash with PBS, additional binding sites were blocked by incubation with BSA (1/% w/v, 300 µl, 1 h, 20-25 °C). Plates were washed two times with PBS and used immediately or stored at 4 °C for up to 7 days. In the assay, sample or standard (50 µl) was added to the wells together with monoclonal antibody (50 µl) at the appropriate dilution (e.g. #3G10B10 at 1/750). After incubation at 20-25 °C for 2 h, wells were washed twice with PBS + 0.05%

Tween® 20 (PBST) and twice with PBS. After incubation at 20-25 °C for 2 h, wells were washed twice with PBS + 0.05% Tween™ 20 (PBST) and twice with PBS. Secondary antibody, horse radish peroxitase conjugated anti-species antibody, e.g. Silenus DAH anti-mouse-HRP (100 µl, 1/2000), was then added to the wells and incubated for 2 h. TMB substrate solution, prepared by adding 110 µl TMB stock (10 mg/ml in DMSO) to 11 ml of sodium acetate buffer (0.1 M, pH 5.5) containing 0.005% H_2O_2, was then added and incubated for 15 minutes. The reaction was stopped by addition of H_2SO_4 (50 µl, 2 M), and the absorbance at 450 nm was determined with a microplate spectrophotometer. Standards were prepared for ELISA by dilution in the methanol in PBS to a maximum methanol concentration of 10% (v/v). All samples were analysed at least in duplicate and over a range of dilutions.

Results of the above-described test are illustrated in Fig. 7.

<u>ELISA method using antibody #3G10B10 in detail</u>

The principle of the indirect competitive microcystin ELISA (MC-ELISA) employed is shown in Fig. 4. This method comprises the following steps:

1. Prepare antigen (OVA-ADDA-HG3199) in bicarbonate buffer, pH 9.6 at 2:5 µg/ml (5 ml/plate).
2. Coat antigen onto microtitre plate at 50 µl/well, tap to mix and cover, incubate overnight at room temperature (RT, 22 °C).
3. Wash two times in PBS, aspirate.
4. Block plate with 1% BSA – (300 µl for 1 hour at RT (22 °C)).
5. Wash two times in PBS, aspirate and use immediately or add 2-300 µl PBS for storage.

   The plates can be stored at this stage in PBS (at 4 °C) for up to 7 days.
6. Add 50 µl sample, or standard, in PBS;

   and 50 µl of antibody **#3G10B10** at 1/750 dilution in BSA-blocker and incubate for 2 hours at RT (22 °C).

   Standard curve: Primary 1000 ng/ml, then nine serial 1:4 dilutions (1 + 3) in PBS.

7. Wash two times in PBS/Tween, two times in PBS.
8. Add 100 µl of secondary antibody conjugate diluted in OVA (Horseradish peroxidase-conjugated rabbit-anti-mouse IgG (Silenus DAH) at a final dilution of 1/2000 and incubate for 2 hours at RT (22 °C).
9. Wash two times in PBST, two times in PBS, aspirate.
10. Turn on plate reader – needs a 15 minute warm up before reading at step 13.
11. Add 100 µl of substrate. Incubate for 15 minutes at RT (22 °C) until colour develops.
12. Add 50 µl stop solution (2M H_2SO_4).
13. Read absorbance at 450 nm. Note that the absorbance at 655 nm can be measured prior to adding the stop solution if required.

Preparation of buffers:

Bicarbonate coating buffer

Dissolve 0.85 g Na_2CO_3, (or 2.15 g $Na_2CO_3 \cdot 2\ H_2O$) and 1.47 g $NaHCO_3$ in 500 ml distilled water, adjust pH to 9.6 (gives 0.05 M bicarbonate).

Phosphate Buffered Saline (PBS)

To prepare 10 times stock solution:

$NaH_2PO_4 \cdot 2\ H_20$	2.897 g (or NaH_2PO_4 anhydrous 2.06 g)
Na_2HPO_4 anhydrous	11.938 g
NaCl	87.660 g

Weigh phosphates, add water to 800 ml, adjust pH to 7.4, then add salt.

Add water to 1 l and check pH (must be 7.2 to 7.6).

Dilute 1/10 for use: gives 0.01 M wrt phosphate and 0.15M NaCl.

Ref.: Mishell et al. (1980)

PBS/Tween

Suspend Tween-20 at 0.05% in PBS (0.5 ml/l);

Use for the washing steps desribed above.

OVA-blocking buffer

Dissolve OVA (Sigma A-5503) in PBS at 1% (2g/200ml).

Use for blocking plates, and as diluent for Ab and Ab".

Secondary antibody

Also referred to herein as Ab", HRP-conjugate, and Second Ab. The dilution depends on the batch used, but approximate dilutions are as follows:

ICN HRP-conjugated rabbit-anti-SHEEP-IgG #55814

Use at a working dilution of 1/3000. Stock solution is stored at 1/10 in PBS thiomersal (0.02%).

TMB Substrate

Prepare stocks of:

1) Sodium acetate buffer 0.1M, pH 5.5 (1.315g/200ml) (check for precipitate before use).

2) TMB (3,3',5,5'-tetramethylbenzidine) at 10mg/ml DMSO; [store in the dark at RT (22°C)].

Immediately before use:

Dissolve 110 µl TMB solution (2) in 11ml sodium acetate buffer (1), and, add 165 µl H_2O_2 (prepared freshly by diluting 38 µl 30% H_2O_2 (commercial strength) into 2.5 ml distilled H_2O).

ELISA Plates

96-well-plates were from NUNC (Maxisorp I plates, catalogue #439454).

Characterization of polyclonal anti-ADDA-antibody developed in sheep

The optimal concentrations of assay reagents were determined empirically by chequerboard titrations. Assay standard curves were calculated using Microsoft Excel. Cutoff values of 20 to 80% of maximum absorbance were used in order to determine the working range. Cross-reactivity of the assay was determined against congeners of MC-LR, -RR, -YR, -LW, -LF, desmethyl-MC-LR, desmethyl-MC-RR and nodularin and calculated from the concentration of analogue giving

50% inhibition (I_{50}) of binding to the protein-ADDA solid phase, expressed relative to the I_{50} for free microcystin-LR. The calculation of the cross-reactivity demonstrates that for sample concentrations ranging between 0.01 and 1 ng/ml the actual toxin concentrations are underestimated in the worst case by 5%. As of a sample concentration ranging betwee 1 ng/ml and 1 µg/l, most congeners tested are detected with equal sensitivity, i.e. 100% cross-reactivity (cf. Fig. 5), while the concentrations of MC-RR and nodularin are slightly overestimated (<5%). This demonstrates that microcystin and nodularin congeners can be detected reliably over a concentration range which is tenfold lower than the safe limit proposed by the WHO.

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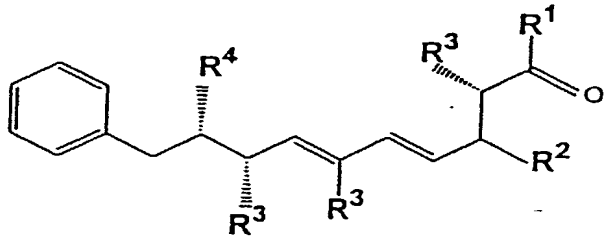
AMENDED CLAIMS PER INTERNATIONAL PRELIMINARY EXAMINATION REPORT DATED 19 JULY 2001

Claims

1. A compound comprising one or more polypeptides providing a binding site of a monoclonal, polyclonal or recombinant antibody or a functionally active derivative or part thereof for a group represented by the following formula (I)



(I)

which is part of a toxin derived from a cyanobacterium, wherein

group R^1 represents a halogen atom, $-OSO_3$, $-OR'$ or $-NR'_2$ and

group R^2 represents hydrogen, (C_1-C_4)alkyl, (C_1-C_4)alkoxy, (C_1-C_4)acyl, (C_1-C_4)aminoacyl or (C_1-C_4)carboxyaminoacyl, or the groups R^1 and R^2 are connected to each other to form a cyclic moiety,

the groups R^3 which may be the same or different are each independently selected from the group consisting of hydrogen and (C_1-C_4)alkyl,

group R^4 represents (C_1-C_4)alkoxy,

and wherein the phenyl group may be substituted or unsubstituted.

2. The compound according to claim 1, wherein the groups R' represent independently from each other hydrogen, substituted or unsubstituted (C_1-C_4)alkyl or (C_1-C_4)acyl, when bound to nitrogen.

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3. The compound according to claim 1 or 2, wherein the groups R^3 each represent methyl and group R^4 represents methoxy.

4. The compound according to any one of claims 1 to 3, wherein group R^1 represents aminoacyl and group R^2 represents (C_1-C_4)acyl.

5. The compound according to claim 4, wherein group R^1 represents glycyl or D-alanyl and group R^2 represents acetyl.

6. The compound according to any one of claims 1 to 5, wherein group R^1 represents $-NH_2$ and group R^2 represents glutamidyl or 2-aminopropionamidyl.

7. The compound according to any one of claims 1 to 6, wherein the toxin is selected from the group consisting of mycrocystin and nodularin congeners.

8. The compound according to any one of claims 1 to 7 which is a polyclonal, monoclonal or recombinant antibody or a functionally active derivative or fragment thereof.

9. A method for the preparation of the compound according to any one of claims 1 to 8 comprising the steps of

(a) preparing a compound containing a group represented by formula (I) as defined in any one of claims 1 to 7,

(b) coupling the compound of step (a) to a carrier.

10. The method according to claim 9, wherein the carrier is a polymeric substance.

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11. The method according to claim 10, wherein the polymeric carrier is selected from the group consisting of polyethyleneglycol, polypeptides, proteins, polysaccharides or plastic supports.

12. The method according to claim 11, wherein the protein carrier is selected from bovine serum albumin, ovalbumin, cationised bovine serum albumin or horseradish peroxidase.

13. The method according to any one of claims 9 to 12 which further comprises the steps of
    (c) immunizing an animal with the conjugate obtained in step (b), and
    (d) isolating the animal's blood, blood serum and/or spleenocytes.

14. A diagnostic kit containing the compound according to any one of claims 1 to 8.

15. An affinity matrix containing the compound according to any one of claims 1 to 8 coupled to a polymeric resin.

16. Use of the compound according to any one of claims 1 to 8 for the detection of a compound containing the group represented by the formula (I).

17. A method for concentrating a compound containing the group represented by the formula (I) from a fluid or for substantially decreasing the amount of a compound containing the group represented by the formula (I) in a fluid com-

AMENDED CLAIMS PER INTERNATIONAL PRELIMINARY EXAMINATION REPORT DATED 19 JULY 2001

prising the steps of

(a) preparing the compound according to any one of claims 1 to 8,

(b) coupling the compound obtained in step (a) to a polymeric matrix, and

(c) contacting the fluid with the polymeric matrix obtained in step (b).

18. The method according to claim 17, wherein the fluid is hemodialysis water, drinking water or water derived from rivers, lakes and oceans.

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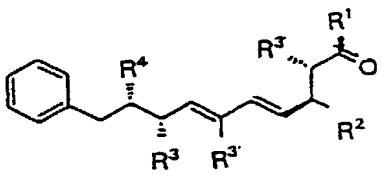
(54) Title: CONGENER INDEPENDENT DETECTION OF MICROCYSTIN AND NODULARIN CONGENERS



(I)

(57) Abstract: The present invention relates to a proteinaceous compound or functionally active derivative or part thereof having a binding site for a group represented by formula (I) which is part of a group of toxins derived from various cyanobacteria, to a method for its production, to diagnostic kits and to an affinity matrix (e.g. for use in immunoaffinity columns, online detection and purifications devices) containing the proteinaceous compound as well as to methods for substantially decreasing the amount of a compound containing the group represented by formula (I) in fluids or for concentrating compounds, e.g. toxins, containing the group represented by formula (I) from fluids such as crude water samples, extracts of algae or other tissue samples, e.g. to determine toxin concentrations.

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Preparation of Anti-ADDA Antibody

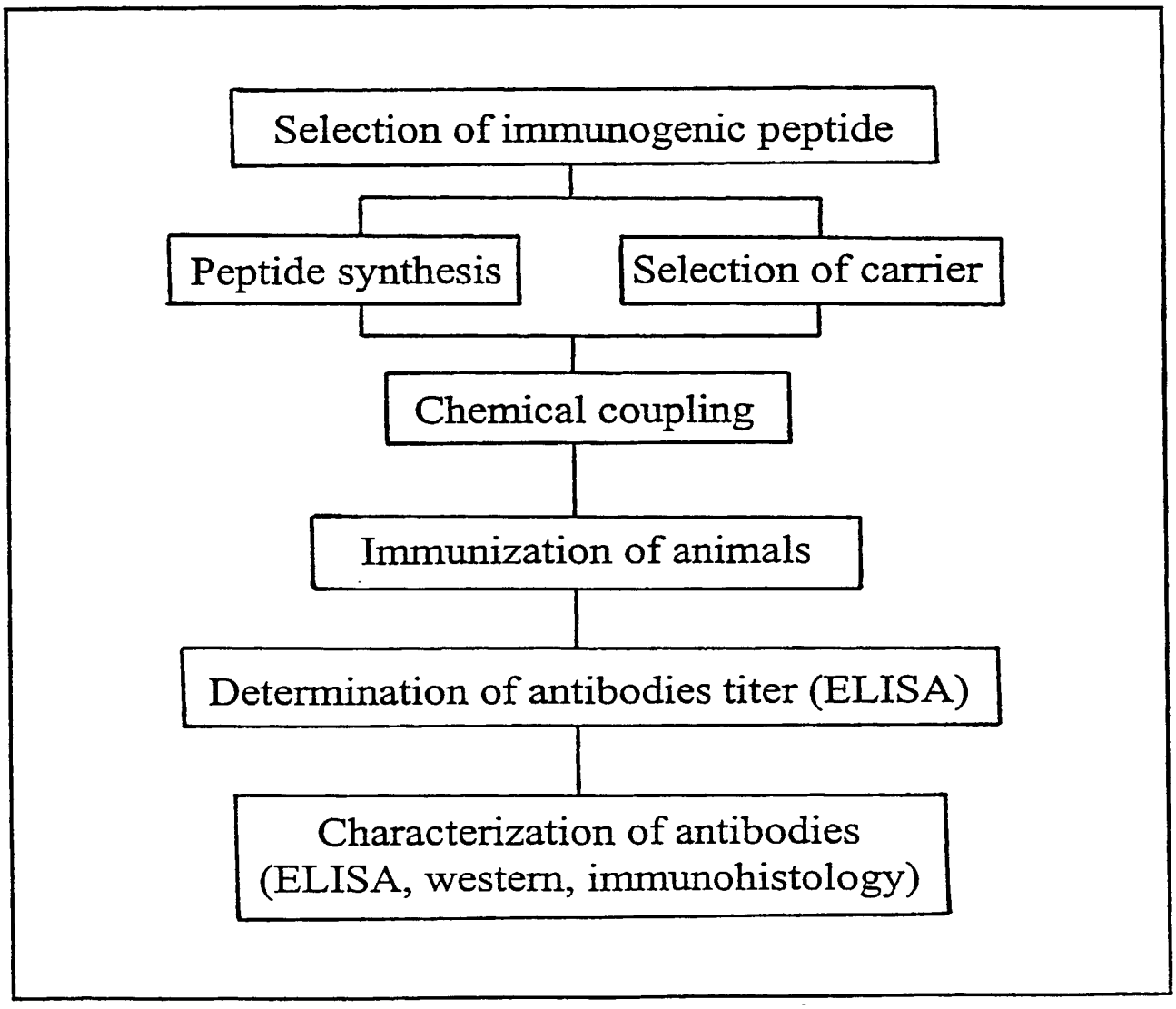
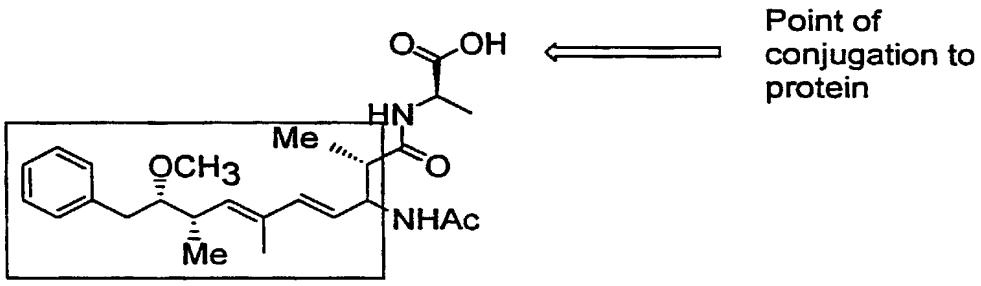
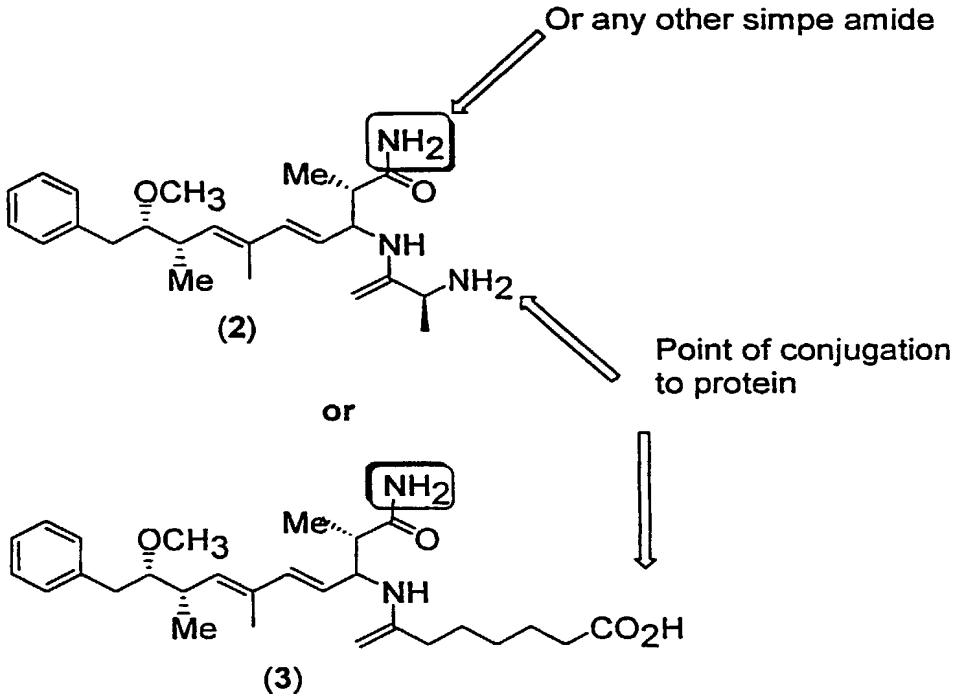


Fig. 1

Antibodies to the region of the molecule enclosed in the box are desired. Therefore, this part has been kept constant and the chemistry which is used to couple the the ADDA-hapten to the protein has to be varied in order to yield a suitable immunogen and antigen



N-Ac-ADDA-Gly-OH (1)



Glutarate derivative of amine

Fig. 2

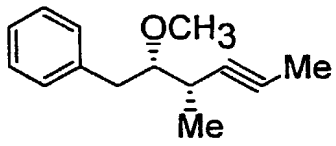
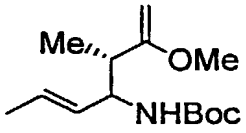
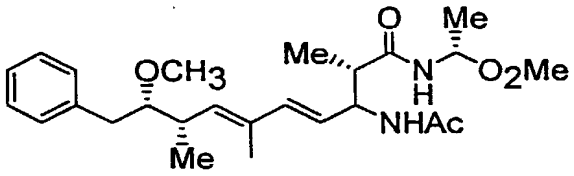
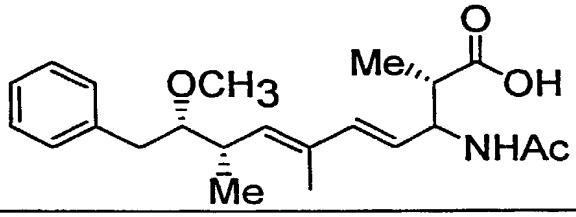
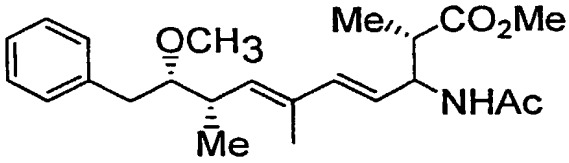
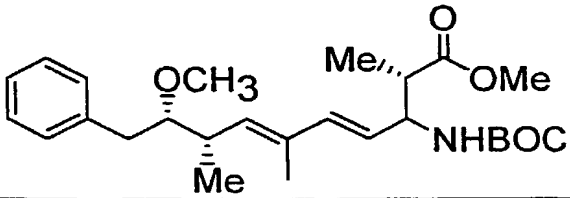
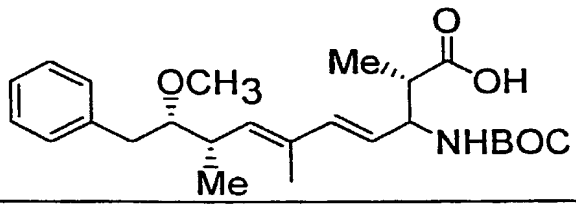
ADDA-Derivatives	
	Alkyne precursor MW 202.32
	"Vinyliodide" precursor MW 369.23
	"ADDA", N-acetyl, D-ala, methyl ester MW 458.65
	"ADDA", N-acetyl, free acid MW 373.54
	"ADDA" N-acetyl, methyl ester MW 387.57
	"ADDA", BOC-amine, methyl ester MW 445.66
	"ADDA", BOC-amine, free acid MW 431.63

Fig. 3

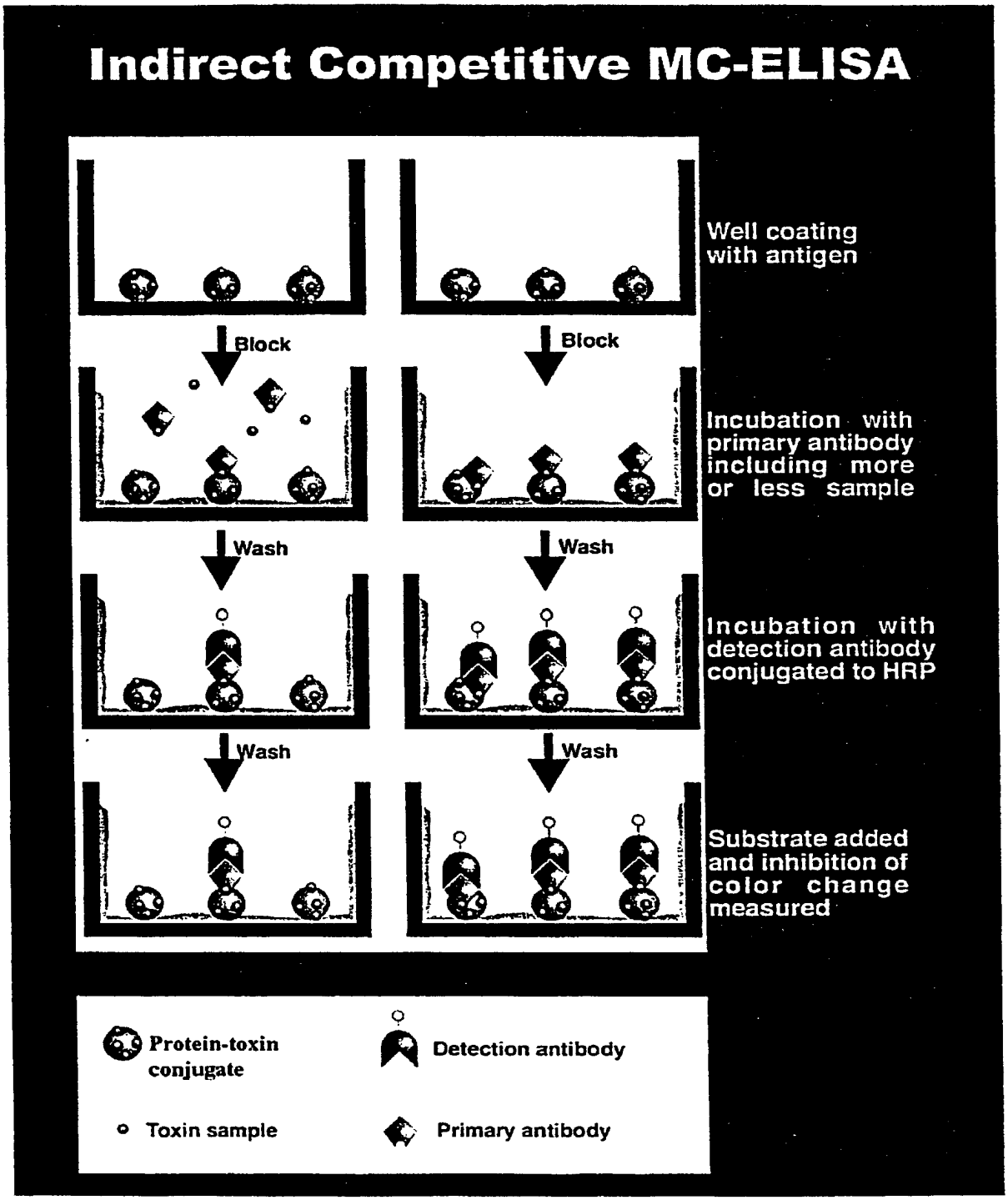

Indirect Competitive MC-ELISA
Well coating with antigen
Block
Block
Incubation with primary antibody including more or less sample
Wash
Wash
Incubation with detection antibody conjugated to HRP
Wash
Wash
Substrate added and inhibition of color change measured
Protein-toxin conjugate
Detection antibody
Toxin sample
Primary antibody

Fig. 4

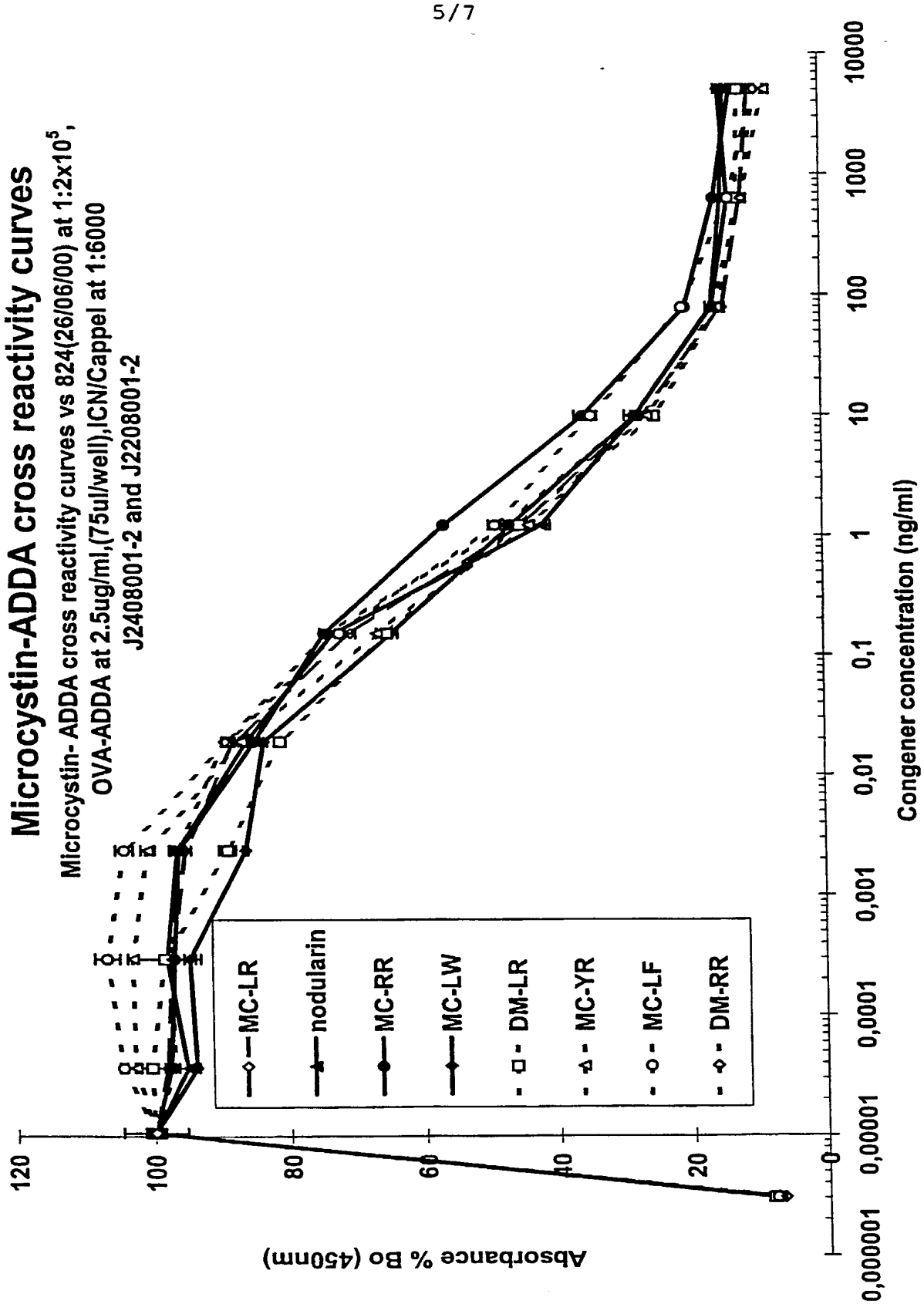

Microcystin-ADDA cross reactivity curves
Microcystin- ADDA cross reactivity curves vs 824(26/06/00) at 1:2x10^5,
OVA-ADDA at 2.5ug/ml,(75ul/well),ICN/Cappel at 1:6000
J2408001-2 and J2208001-2
Absorbance % Bo (450nm)
Congener concentration (ng/ml)
MC-LR
nodularin
MC-RR
MC-LW
DM-LR
MC-YR
MC-LF
DM-RR

Fig. 5

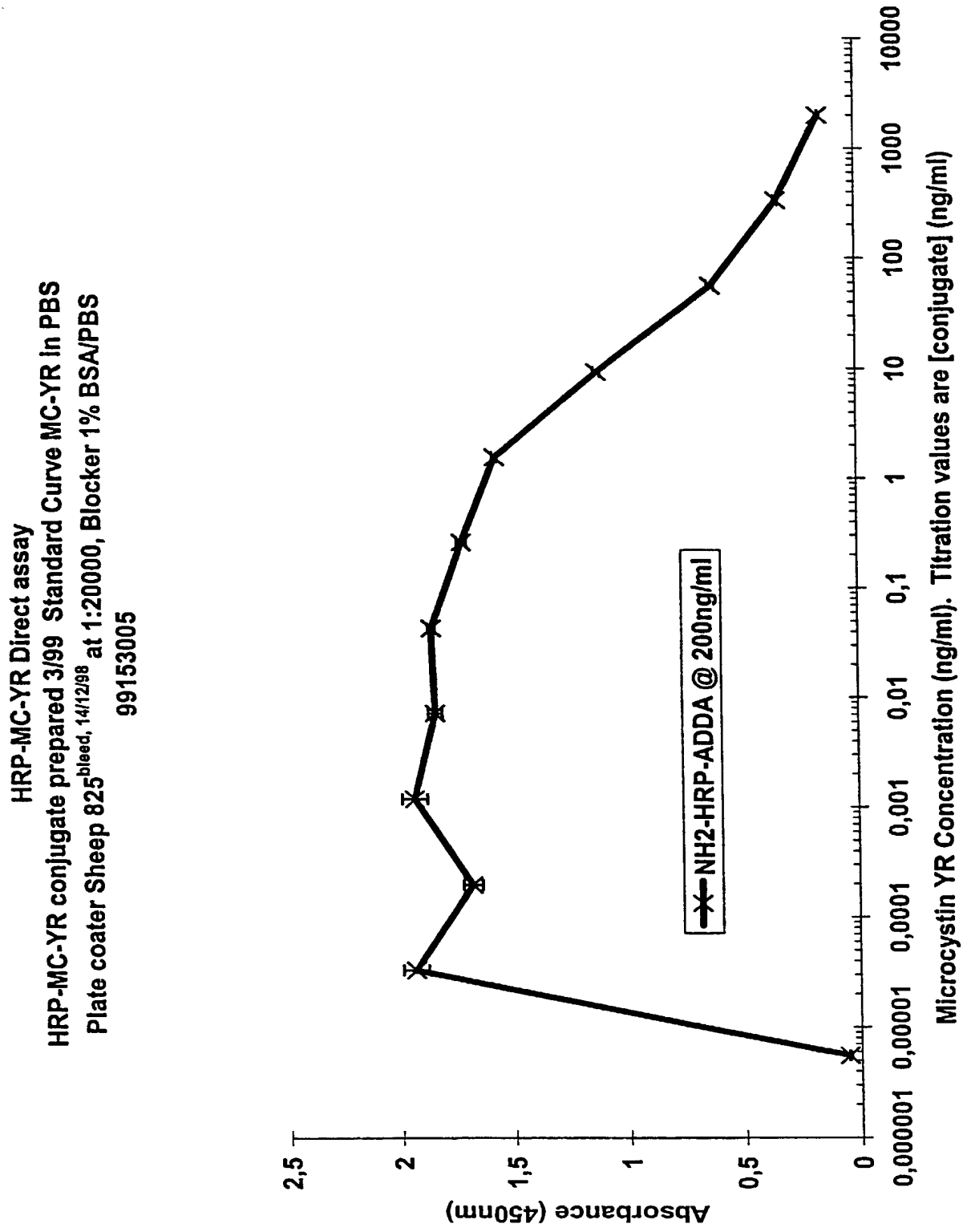

HRP-MC-YR Direct assay
HRP-MC-YR conjugate prepared 3/99 Standard Curve MC-YR in PBS
Plate coater Sheep 825bleed, 14/12/98 at 1:20000, Blocker 1% BSA/PBS
99153005
NH2-HRP-ADDA @ 200ng/ml
Absorbance (450nm)
2,5
2
1,5
1
0,5
0
0,000001 0,00001 0,0001 0,001 0,01 0,1 1 10 100 1000 10000
Microcystin YR Concentration (ng/ml). Titration values are [conjugate] (ng/ml)

Fig. 6

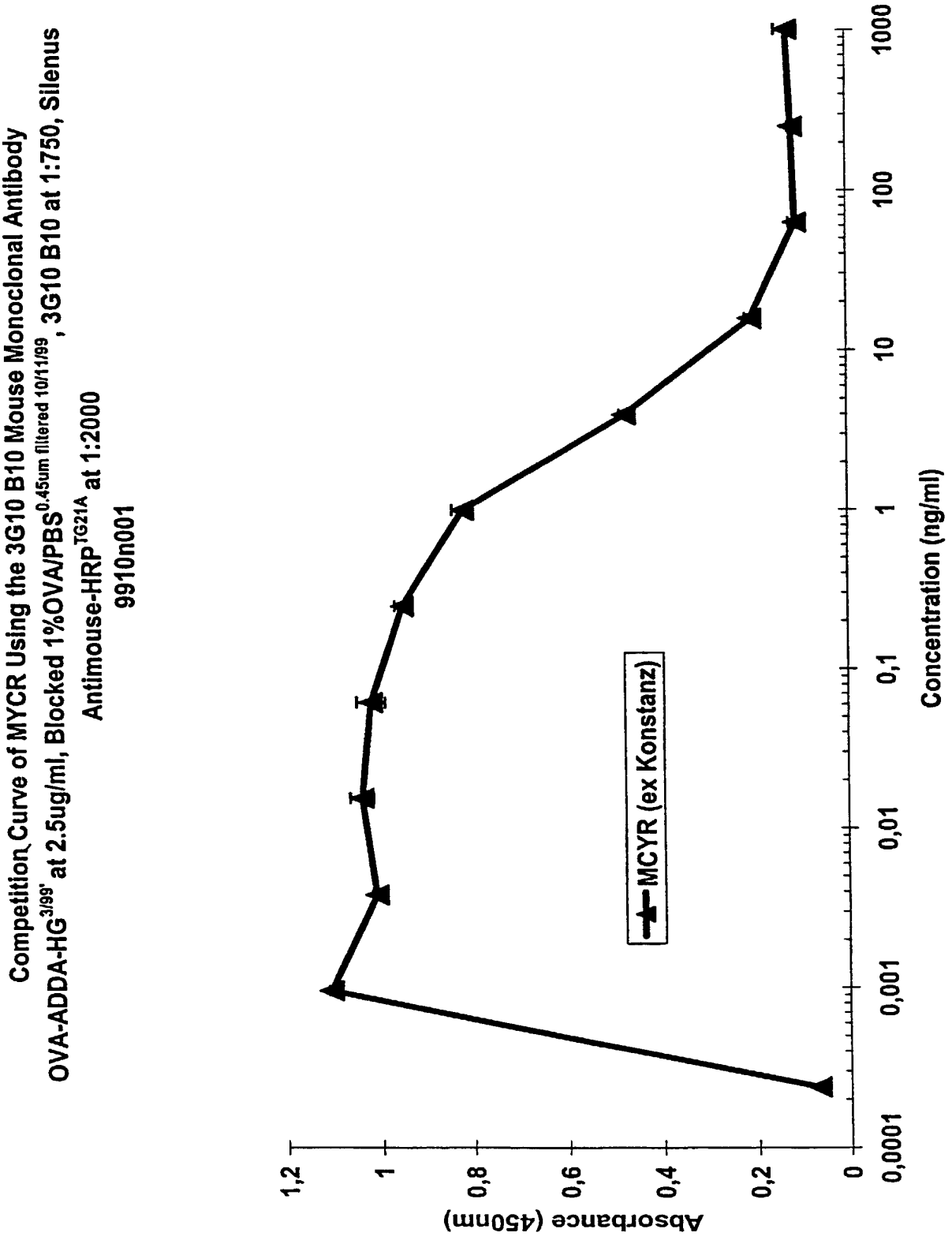

Competition Curve of MYCR Using the 3G10 B10 Mouse Monoclonal Antibody
OVA-ADDA-HG3/99' at 2.5ug/ml, Blocked 1%OVA/PBS0.45um filtered 10/11/99, 3G10 B10 at 1:750, Silenus
Antimouse-HRPTG21A at 1:2000
9910n001
MCYR (ex Konstanz)
Absorbance (450nm)
1,2
1
0,8
0,6
0,4
0,2
0
0,0001
0,001
0,01
0,1
1
10
100
1000
Concentration (ng/ml)

Fig. 7

Attorney
Docket No.: MBP-010Xx

DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

CONGENER INDEPENDENT DETECTION OF MICROCYSTIN AND NODULARIN CONGENERS

The specification of which (check one):

[ ] **is attached hereto.** [X] **was filed on** March 5, 2002 **as Application No.** 10/070,302 ; **amended on** _____ **(if applicable).**

[X] was filed as PCT International. Appl. No. PCT/EP00/08711 on 06 September 2000, and was amended under PCT Article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

I hereby claim foreign priority benefits under Title 35, USC §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)		Date Filed	Priority Claimed	
99 116 881.6 (Number)	Europe (Country)	06 September 1999 (Day/Month/Year)	[X] Yes	[ ] No
_____ (Number)	_____ (Country)	_____ (Day/Month/Year)	[ ] Yes	[ ] No
_____ (Number)	_____ (Country)	_____ (Day/Month/Year)	[ ] Yes	[ ] No

I hereby claim the benefit under Title 35, USC §119(e) of any United States provisional application(s) listed below:

_____ (Application Number)	_____ (Filing Date)
_____ (Application Number)	_____ (Filing Date)
_____ (Application Number)	_____ (Filing Date)

Express Mail Number

EV 009950215 US

Attorney
Docket No.: MBP-010XX

I hereby claim the benefit under Title 35 USC §120 of any United States application(s) listed below and insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35 USC §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application No.)	(Filing Date)	(Patented/pending/abandoned)
(Application No.)	(Filing Date)	(Patented/pending/abandoned)
(Application No.)	(Filing Date)	(Patented/pending/abandoned)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) to prosecute this application and transact all business connected therewith in the Patent and Trademark Office, and to file with the USRO any International Application based thereon.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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